AD_____

Award Number: DAMD17-00-1-0604

TITLE: Is the Regenerative Capacity of the Mammary Gland Contained with those Mammary Cells that Express the Progesterone Receptor? Implications for Breast Cancer

PRINCIPAL INVESTIGATOR: John P. Lydon

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: September 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | September 2001 | Final (1 Sep 00 - 31 Aug 01) |

**4. TITLE AND SUBTITLE**
Is the Regenerative Capacity of the Mammary Gland Contained with those Mammary Cells that Express the Progesterone Receptor? Implications for Breast Cancer

**5. FUNDING NUMBERS**
DAMD17-00-1-0604

**6. AUTHOR(S)**
John P. Lydon

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Baylor College of Medicine
Houston, Texas 77030

E-Mail: jlydon@bcm.tmc.edu

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**
Report contains color

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 Words)*

Recent transgenic studies have demonstrated that targeted overexpression of the A and B isoforms of the progesterone receptor (PR) to the mammary gland results in excessive ductal branching and alveolar growth respectively. Based on the above, we hypothesized that mammary-specific stem cells are present as a subgroup within those epithelial cells that express PR. To determine whether a stem cell population is contained within the PR expressing cell population ($PR^+$), a PR-lacZ knockin mouse in combination with fluorescent-activated cell sorting (FACS) was utilized to separate $PR^+$ from $PR^-$ mammary cells; both cell populations would then be evaluated for their regenerative capacity by transplantation into the cleared mammary fat pad of a host animal. FACS analysis applied to the PR-lacZ mammary gland provided a significantly enriched (~70%) $PR^+$ mammary epithelial cell population that maintained lacZ expression in culture. Although, $PR^+$ and $PR^-$ enriched mammary epithelial cell populations were transplanted into mammary fat pads, these host animals were lost to Tropical Storm "Allison"; estimation of the regenerative potential of the above mammary epithelial cell populations will have to await the expansion of the PR-lacZ colony.

**14. SUBJECT TERMS**
Progesterone Receptor, LacZ Knockin, Stem Cell

**15. NUMBER OF PAGES**
9

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

# INTRODUCTION:

*SUBJECT:* Because transplanted mammary epithelial cells can regenerate typical ductal and alveolar structures in the host mammary fat pad, the existence of a mammary stem cell population has been postulated (1). Using a progesterone receptor (PR) knockout (PRKO) mouse model, we demonstrated that functional PR is essential for pregnancy-induced mammary epithelial ductal proliferation and lobuloalveolar differentiation but not for immediate post pubertal ductal morphogenesis (2). Furthermore, recent transgenic studies have demonstrated that targeted overexpression of either the A or B isoforms of the PR to the mammary gland results in excessive ductal branching for the PR-A transgenic and extensive alveolar growth for the PR-B transgenic (3,4). Collectively, these observations implied that P-signaling pathways may specify the fate of a mammary epithelial cell to become either a ductal or alveolar cell type in the adult. Based on the above, we hypothesized that mammary-specific stem cells are present as a subgroup within those epithelial cells that express both ER and PR.

*PURPOSE AND SCOPE:* To determine whether stem cells represent a subgroup of cells within the PR expressing cell population ($PR^+$), a recently generated PR-LacZ mouse model in combination with fluorescent-activated cell sorting (FACS) was utilized to separate $lacZ^+$ (or $PR^+$) from $lacZ^-$ (or $PR^-$) mammary cells. The final stage of this proposal was to evaluate the regenerative capacity of both isolated cell populations through transplantation into host mammary fat pads devoid of an endogenous epithelial cellular compartment. Our hypothesis predicts that, unlike the $PR^-$ population, $PR^+$ cells would regenerate the mammary ductal tree.

# BODY:

Murine mammary epithelial cells were isolated from 16-week-old nulliparous PR-LacZ females according to the procedures described in (5). Panel A in Figure 1 shows a typical lacZ stained mammary gland whole mount from these mice; panel B represents a higher magnification of panel A revealing the nonuniform organization of epithelial cells that express the PR. Panel C depicts a section through a ductal structure revealing that PR expression is limited to the luminal epithelial component of the gland; see black arrow. Because the success of this "CONCEPT" proposal is predicated upon isolating viable $PR^+$ cells that still exhibit lacZ expression in culture, whole mammary cell cultures derived from the PR-lacZ mouse were initially evaluated for lacZ expression. Panel D demonstrates that cellular lacZ expression is maintained in culture and furthermore that this expression is preserved following multiple passaging of the cells (see white arrows).

Exploiting lacZ encoded β-galactosidase activity within $PR^+$ epithelial cells, FACS analysis was employed to isolate $PR^+$ from $PR^-$ mammary epithelial cells. Briefly, from 5 mice (both inguinal glands/mouse with lymph nodes removed)), epithelial cells were isolated and hypotonically permeabilized with the following staining solution: (in 100 μl) 1X PBS containing 4% (v/v) fetal calf serum, 10 mM HEPES, pH 7.2 and the fluorogenic substrate: 2mM fluorescein di-β-D-digalactopyranoside (FDG; (F-1179) Molecular Probes, Inc.) and incubated at 37°C for 1 minute. The incubation was terminated by incubating on ice for 5 min before being diluted to 1 ml with the above staining solution. Due to the severity of the loading method, cells were rapidly pelleted by centrifugation

and resuspended in 2ml of staining solution containing propidium iodide (5ug/ml) to later identify those cells that did not survive the procedure.

Using the Flow Cytometry Core Facility at Baylor College of Medicine, cells were sorted using an argon laser (488nm) to excite the two fluorochromes: propidium iodide (to detect dead cells) and fluorescein (to detect $lacZ^+$ cells) and collected at band paths 610nm and 525 nm respectively. Panels E (wild type mice: negative control) and F (PR-LacZ mice) show a typical two-dimensional contour plot of scattered cells according to the fluorescence intensities of propidium iodide (PI) (quadrants M1 and M2-dead cells) and the final hydrolyzed product of FDG: fluorescein (quadrant M4); quadrant M3 denotes viable cells that are $lacZ^-$. Panel E, representing wild type, shows that approximately 80% of the cellular population were viable; 15% of the cellular population did not survive the FDG loading procedure, whereas ~4% of the total cell population exhibited background fluorescence. In the case of PR-lacZ mice, ~56% of the total cell population were viable $PR^-$ mammary cells; approximately 19% of the cell population did not survive the treatment protocol whereas ~25% of the total cell population exhibited fluorescein-derived fluorescence. As indicated in Panel F, a region representing maximum viability and cell number were selected (gated) in quadrants M3 and M4 for subsequent cellular-transplantation into epithelial deficient mammary fat pads of host animals. Panels G and H represent cytospin preparations from quadrants M3 and M4 respectively, shown in panel F. Due to cellular "clumping", panel H demonstrates that a pure $lacZ^+$ cellular population was not obtained, usually 70-80% this cellular population was $LacZ^+$. Although $lacZ^-$ and $lacZ^+$ (70% enriched) cell populations were transplanted into donor fat-pads, mice harboring these recombinant glands, including most of the PR-lacZ colony, were lost to Tropical Storm "Allison" in June 2001; we are currently rebuilding the colony with a view to completing this experiment in the future.

## KEY RESEARCH ACCOMPLISHMENTS:

Exploiting a novel PR-lacZ mouse in combination with FACS analysis, we were able to isolate a significantly enriched mammary epithelial cellular population that expresses the PR. In addition to providing cellular material for the studies proposed in this CONCEPT AWARD, future studies may utilize these cell populations in conjunction with high density DNA microarray technology to detect signaling pathways downstream of the PR.

## REPORTABLE OUTCOMES:

The data described herein will be presented as preliminary data for an IDEA AWARD in 2002, sponsored by the Department of Defense.

## CONCLUSIONS:

Due to extenuating circumstances (described in the **BODY**), it was not possible to address the final goal of this proposal, namely to evaluate the regenerative capacity of the individual PR-$lacZ^-$ and PR-$lacZ^+$ cell populations; however, the studies described herein provide support that the original CONCEPT is feasible. Finally, the targeted insertion of the autofluorescent green fluorescent protein into the PR gene may provide

an even more effective separation of $PR^+$ from $PR^-$ cells than obtained with the PR-lacZ mouse; this mouse is currently being generated.

## REFERENCES:


1. Chepko G, Smith GH. Mammary Epithelial Stem Cells: Our Current Understanding. Journal of Mammary Gland Biology and Neoplasia. 1999; 4:35-52.

2. Lydon JP, Ge G, Kittrell FS, Medina D, O'Malley BW. Murine Mammary Gland Carcinogenesis is Critically Dependent on Progesterone Receptor Function. Cancer Research. 1999; 59: 4276-4284.

3. Shyamala G, Yang X, Silberstein G, Barcellos-Hoff MH, Dale E. Transgenic mice carrying an imbalance in the native ratio of A to B isoforms of progesterone receptor exhibit developmental abnormal mammary glands. Proceedings of the National Academy of Sciences, USA. 1998; 95 : 696-701.

4. Shyamala G, Yang X, Cardiff RD, Dale E. Impact of Progesterone Receptor on Cell-Fate Decisions during Mammary Gland Development. Proceedings of the National Academy of Sciences, USA. 2000; 97: 3044-3049.

5. Smith GH, Gallahan D, Zweibel JA, Freeman SM, Bassin RH, Callahan R. Long-Term In Vivo Expression of Genes Introduced by Retrovirus-Mediated Transfer into Mammary Epithelial Cells. Journal of Virology. 1991; 65: 6365-6370.

# APPENDIX

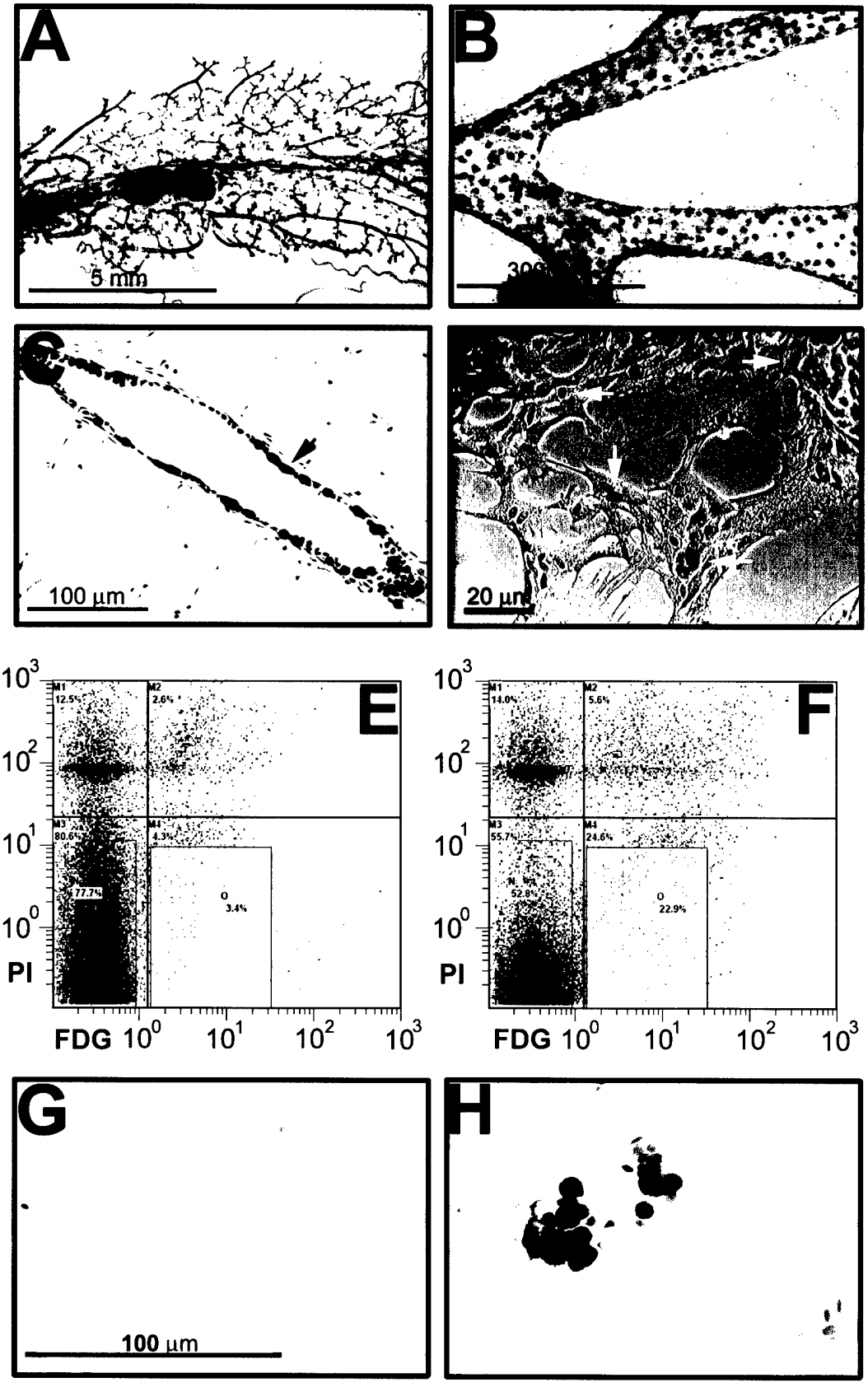
A
5 mm
B
C
100 µm
20 µm
E
F
PI
FDG
G
100 µm
H

**FIGURE 1. FACS analysis of the PR-lacZ mammary gland.**
Panels A and B represent whole mounts of lacZ stained mammary glands from adult virigin mice; note the non-uniform expression pattern for PR in the main ducts (Panel B). Panel C represents a transverse section through a typical duct shown in (B); PR expression is limited to the luminal epithelial cellular component. Panel D represents lacZ staining of cultured mammary cells (epithelial, myoepithelial, and fibroblast cells) isolated from the PR-lacZ mammary gland; lacZ expression was maintained through a number of passaging steps. Panels E (wild type mice) and F (PR-lacZ mouse) represent two dimensional contour plots of scattered viable and nonviable $PR^-$ and $PR^+$ lacZ mammary epithelial cells. Panels G and H show lacZ stained mammary epithelial cells from the quadrants M3 and M4 shown in Panel F respectively; note the significant enrichment of $PR^+$ cells in Panel H.